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Co-evolution of segregation guide DNA motifs and the FtsK translocase in bacteria: identification of the atypical *Lactococcus lactis* KOPS motif

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ABSTRACT

Bacteria use the global bipolarization of their chromosomes into replichores to control the dynamics and segregation of their genome during the cell cycle. This involves the control of protein activities by recognition of specific short DNA motifs whose orientation along the chromosome is highly skewed. The KOPS motifs act in chromosome segregation by orienting the activity of the FtsK DNA translocase towards the terminal replichore junction. KOPS motifs have been identified in γ-Proteobacteria and in *Bacillus subtilis* as closely related G-rich octamers. We have identified the KOPS motif of *Lactococcus lactis*, a model bacteria of the *Streptococcaceae* family harbouring a compact and low GC% genome. This motif, 5'-GAAGAAG-3', was predicted in silico using the occurrence and skew characteristics of known KOPS motifs. We show that it is specifically recognized by *L. lactis* FtsK in vitro and controls its activity in vivo. *L. lactis* KOPS is thus an A-rich heptamer motif. Our results show that KOPS-controlled chromosome segregation is conserved in *Streptococcaceae* but that KOPS may show important variation in sequence and length between bacterial families. This suggests that FtsK adapts to its host genome by selecting motifs with convenient occurrence frequencies and orientation skews to orient its activity.

INTRODUCTION

Bacterial chromosomes are large, usually circular, DNA molecules that replicate from a unique origin (ori) and in a bidirectional manner to the opposite termination region (ter). This replicative organization is accompanied by a global ori-ter polarization of chromosome sequences that now appears as the most general and conserved feature of bacterial genome organization and dynamics ([1,2] for reviews). The term ‘replichore’ has been coined to account for this ori-ter polarization (3).

Replichores are characterized by an asymmetric base composition (the GC-skew), with the leading strands being richer in guanine than lagging strands (4), and by an orientation bias of numerous DNA motifs (5,6). Of these, two have been shown to have a biological function: the chi sites that protect chromosomal DNA against degradation and promote homologous recombination ([7] for review) and the KOPS motifs that act in chromosome segregation by controlling the activity of the FtsK protein (8,9). Both chi and KOPS are over-represented in genomes (i.e. their occurrence is significantly higher than expected by chance). Their enrichment on leading strands (here referred to as leading strand skew or skew) is also significant (8,10), which means that they are more skewed than expected even when taking into account the GC skew. Depending on the phylum, chi sites are recognized by analogous systems such as RecBCD (11) or AddAB/RexAB (12,13). Consistently, although their distribution properties among bacterial genomes are conserved, known chi sites vary in length and sequence in several Proteobacteria and Firmicutes (10,14,15).

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

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In contrast to the RecBCD/AddAB systems, most bacteria possess an FtsK orthologue (16,17). In *Escherichia coli*, FtsK acts both in chromosome segregation and cell division and is thought to couple these during the cell cycle [for reviews (16,18)]. Its N-terminal domain, as part of the cell division apparatus, targets FtsK to the division septum. Its C-terminal domain, FtsKC, is the most conserved part of the protein and forms a dsDNA-translocase of the AAA+ ATPase family (19,20). FtsKC acts in the terminal region of the chromosome (21) and controls the last steps of segregation including the removal of catenation links between sister chromosomes and the resolution of chromosome dimers (22,23). FtsKC assembles as a hexameric motor upon interaction with the DNA (24). This interaction may occur with non-specific DNA but is preferential with KOPS motifs, which orient translocation in the direction specified by KOPS (24–26). KOPS are recognized by a winged-helix DNA-binding domain located in the extreme C-terminal FtsKγ subdomain (27). The crystal structure of a KOPS motif bound to *E. coli* or *Pseudomonas aeruginosa* FtsKγ revealed that three FtsKγ subdomains of the six present in an FtsKC hexamer are involved in the recognition of a single KOPS motif (26). Once assembled onto the DNA, FtsK translocates towards the terminal junction of KOPS polarity, at which the *dif* recombination site lies and finally activates XerCD-mediated recombination between *dif* sites to resolve chromosome dimers (28,29).

Most FtsK orthologues contain a conserved FtsKγ subdomain, suggesting that the KOPS-mediated control of FtsK translocation is conserved (16). Few data are, however, available for conservation of the KOPS motif. The proposed consensus for *E. coli* KOPS, 5'-GGGNNAGGG-3' (8), contains the 5'-GGGCAGGG-3' motif that is also recognized by the γ-Proteobacteria *Vibrio cholerae* and *P. aeruginosa* FtsK homologues (26,30). SpoIIIE, an FtsK homolog of the Firmicute *B. subtilis*, does not recognize this motif but the 5'-GAGAAGGG-3' motif (the SRS motif), equivalent to KOPS in length and only slightly divergent in sequence (31). This sequence conservation between KOPS motifs in phylogenetically distant species may suggest that KOPS/SRS represent prototypical motifs with conserved function in a wide range of bacterial phyla. A global search for skewed octamers whose skew increases towards the terminal region (called Architecture Imparting Sequences, AIMS) was conducted in 40 bacterial genomes (6). The 5'-GGGCGAGGG-3' motif in *E. coli* and 5'-GAGAAGGG-3' motif in *B. subtilis* responded to these criteria. However, whereas the 5'-GGGCAGGG-3' displays AIMS characteristics in most Proteobacteria, no common motif was identified in Firmicutes, suggesting that different and/or more criteria are needed to predict KOPS or the KOPS motifs can diverge in sequence inside a bacterial phylum.

*Lactococcus lactis* is a mesophilic lactic acid bacteria extensively used in dairy and health applications. Due to its industrial importance, it serves as a model organism for genetic and biochemical studies of this group of micro-organisms. Phylogenetically, *L. lactis* constitute the first branch that separates the *Streptococcaceae* from other Firmicutes. We have previously shown that *Streptococcaceae* possess an atypical Xer system, the XerS/difSL system, that uses a single recombinase, XerS, instead of the two XerC and XerD recombinase of classical Xer systems, and a divergent *dif* site for chromosome dimer resolution (32). Despite this difference, resolution of chromosome dimers by XerS/difSL requires the chromosome translocation activity of FtsK (29,32). We now report that the *L. lactis* chromosome contains KOPS motifs that orient the activity of FtsK. This motif, 5'-G AAGAAG-3', differs from previously reported KOPS motifs both in sequence and length.

**MATERIALS AND METHODS**

**Strains and plasmids**

Strains used were derived from *E. coli* K12 strain LN2666 [W1485 F' leu thyA thi deoB or C supE rpsL (StrR)] (33). Strains carrying the *dif-lacI-dif* cassette flanked by KOPS motifs were previously described (29). Strains carrying the 5'-GAAGAAG-3' were constructed in a similar manner. The 3γ constructs were designed as genes encoding repeats of FtsKγ subdomains (from residue 1266 of *E. coli* FtsK and residue 693 of *L. lactis* FtsK) separated by a 14 glycine-rich flexible linker (Figure 2A) followed by GT residues (KpnI restriction site) before the second, and HM residues (NdeI restriction site) before the third FtsKγ copy. These constructs were ordered from GenScript (Piscataway, NJ, USA). For protein production and purification, the 3γ genes were inserted into plasmid pFSKB3X (GTP technology, Toulouse, France), creating His-FLAG-3γ fusion genes in plasmids pCL380 (His-FLAG-3γEc) and pCL381 (His-FLAG-3γLc). For in vivo expression, relevant genes were inserted into a pgB2 (34) derivative carrying araC-araBADp expression cassette, yielding plasmids pCL374 (His-FLAG-3γEc) and pCL375 (His-FLAG-3γLc). XerC was produced in vivo from plasmid pFC241 [pGB2-araBADp-xerC; (29)].

**Purification of 3γ proteins**

*Escherichia coli* strain BL21(DE3) carrying plasmid pCL380 or pCL381 was grown in 1-broth at 42°C to OD600 = 0.6. IPTG (0.1 mM) were added to the medium and incubated culture at 25°C for 3 h. Cells were recovered by centrifugation resuspended in buffer [50 mM phosphate buffer pH 8, 500 mM NaCl, 10 mM imidazole, 10% glycerol, 1 mg/ml lysozyme, 230 mg/ml RNaseA and EDTA-free proteases inhibitor cocktail (Roche)] and sonicated, and the lysate was cleared by centrifugation. His-FLAG-tagged 3γ proteins were purified on two successive nickel resin columns (1 ml His-trap HP, GE Healthcare) followed by a gel filtration columns (High-load 16/60 Superdex 200, GE Healthcare). Purified proteins were stored at −80°C in buffer containing 50 mM Hepes (pH 7.8), 40 mM KCl and 0.5 mM EDTA glycerol 10%.
ITC experiments

ITC experiments were performed using a MicroCal ITC200 Isothermal Titration Calorimeter. Experiments were carried out by titrating 3γ protein (50 μM) with DNA fragment as indicated [28 injections of 1.5 μL DNA solution at 450 μM (Figure 4D) or 250 μM (Figure 4E and F) in 3 s with a spacing of 180 s]. The stoichiometry of binding was obtained by fitting the ITC titration curves to the ‘one set of site’ model, assuming that the binding events were equivalent in the case of multiple binding. The best fitting model curves with corresponding stoichiometry are shown in Figure 4D and E.

EMSA experiments

Oligonucleotides were 5’ end-labelled using [γ-32P] ATP and T4 DNA polynucleotide kinase and purified on MicroSpin G-25 column (GE Healthcare). DNA substrates were then prepared by hybridization of complementary labelled and unlabelled oligonucleotides. After 10 min denaturation in boiling water, the mixture was left to slowly cool to 25°C. Binding reactions were done in buffer containing 25 mM Hepes (pH 7.7), 40 mM KCl, 0.25 mM EDTA, 0.5 mM DTT, 10 μg/ml BSA, 10 mM MgCl2 and 10% glycerol, in the presence of 5000 c.p.m of labelled DNA (~10 nM), and when indicated 1 μg of poly(dI-dC) and 0.5 and 1 μM of protein. The reactions were incubated at 25°C for 5 min and analysed on 5% native TBE PAGE. Gels were dried and analysed using a Fuji PhosphorImager.

XerCD/dif recombination assay

Recombination was measured as described in (21,29). Briefly, strains carrying the Δ(xerC::Gm) mutations, an insertion of the dif-lacI-dif cassette and a plasmid producing the γ or 3γ proteins were grown in LB broth plus 0.025% arabinose, rendered competent and transformed with pFC242 (XerC). Transformants were plated on LB-agar containing 20 μg/ml spectinomycin plus 100 μg/ml ampicillin and 0.025% arabinose and grown overnight at 37°C. Five independent transformants were inoculated in the same medium, grown for 5 h, diluted and placed in L broth plus X-gal (40 μg/ml). The ratio of dark blue to total colonies was used to calculate the frequency of lacI loss per generation. The mean and standard deviation of the 5 independent measures is plotted in the figures.

Genome analyses

Genome sequences were extracted from the Genome Review database with the following genome accession numbers: AE003852_GR.1 (V. cholerae chromosome 1), AE005176_GR.1 (L. lactis IL1403), AE007317_GR.1 (Streptococcus pneumoniae R6), AE014074_GR.1 (Streptococcus pyogenes MGA315), AL009126_GR.3 (B. subtilis 168), AL732656_GR.1 (Streptococcus agalactiae NEM316), AM406671_GR.1 (L. lactis spp. Cremoris MG1363) and U00096_GR.2 (E. coli MG1655).

In all species, the analyses were carried on the leading strand as it is the relevant strand for KOPS/SRS activity. Leading strands were defined as the DNA strand reported in Genbank files downstream of the replication origin up to dif and the reverse complement strand from dif to the origin. In B. subtilis, we used the PLR position instead of the origin because it is reported that the skew of SRS shifts in this region and not at the origin (31). Ori/PLR and dif positions were, respectively, 3923 767 and 1 588 801, 1 and 1 564 104, 3 965 606 and 1 942 543 in E. coli, V. cholerae and B. subtilis. For Streptococceae, the origin is at position 1 and the position of dif is 1238 253 (L. lactis MG1363) (32), 1259 289 (L. lactis IL1403), 1009 512 (S. agalactiae NEM316), 1039 995 (S. pneumoniae R6) and 893 748 (S. pyogenes MGA315). The experimentally defined region of FtsK activity around dif (21) includes positions 1438 to 1776 kb that represents roughly 7% of the E. coli chromosome. For the analyses, we used in all species, a region representing 7% of the genome centred on dif that we call in this paper dif region.

Over-representation. Motif count analyses were performed on the leading strand of each strain. Since KOPS have a degenerate nucleotide, we analysed ‘motifs families’ counts: for example, the motif GGGNAGGG is represented by the family GGGAGGG, GGCGAGG, GGGGAGGG and GGGTAGGG. To assess over-representation of motifs of a given length, the observed count of each motif was compared to the count expected in random sequences showing the same oligonucleotide composition. The significance of the difference between the counts was evaluated by calculating the associated P-value, which is the probability that the count of a given motif in a random sequence under a Markov model of order 2 (see Supplementary Material) is greater than the observed count for this motif. The P-value was obtained using a compound Poisson approximation of motif counts (35), which has been shown to be reliable even when the sequence length is relatively short (as is the case for analyses in the dif region).

Skew significance. We define the leading strand skew of a motif as the number of its occurrences on the leading strand of the replication fork over the total number of its occurrences, in the observed region. The statistical significance of the motifs leading strand skew was evaluated by calculating the associated P-value, which is the probability that the skew of a given motif in a random sequence (under a Markov model of order 1, see below) is greater than the observed skew. The P-value was obtained using a Gaussian approximation of motif counts (10).

Orders of the Markov models used for score calculation. We determined empirically the order of the Markov model to use for evaluation of the over-representation score. We compared the rank of the KOPS motif scores in E. coli, B. subtilis and V. cholerae (chromosomes 1) in all possible models (Supplementary Figure S6). We chose the lowest order model (this insures good sampling of the model parameters) that minimized the ranks (thus showing over-representation of the motifs). We retained a Markov model of order 2
for the over-representation score, which is the minimal model to take into account codon bias. For the skew score, we used a model of order 1, which is sufficient to take into account the G/C skew. All calculations were performed using the RMES software (User guide: Hoebeke, M. and Schbath, S. (2006), “R’MES: Finding Exceptional Motifs”, version 3. http://genome.jouy.inra.fr/ssb/rmes) and custom Perl scripts that are available upon request.

RESULTS AND DISCUSSION

Covalent trimers of FtsKγ bind KOPS

Most FtsK homologues, including in L. lactis, contain a domain homologous to the E. coli FtsKγ subdomain in sequence and length located at their C-terminal end (Figure 1A), suggesting a conserved role in DNA binding and the control of translocation. We thus attempted to characterize KOPS motifs using the DNA binding activity of purified E. coli and L. lactis FtsKγ subdomains. However, the poor affinity of purified E. coli FtsKγ subdomain (γEc) to DNA containing KOPS motifs, and the fact that it can be detected in EMSA experiments only in the absence of competitor DNA complicated this approach ([27], data not shown). Since three γEc monomers are involved in the interaction with a single KOPS in the γEc/KOPS co-crystal structure (26), we reasoned that chimera protein containing three FtsKγ might bind KOPS with a higher affinity than FtsKγ monomers. We constructed a gene coding for a chimera protein, 3γEc, that contains three E. coli γ subdomains separated by linkers rich in glycine residues (Figure 1B), predicted to be flexible and already successfully used for the construction of covalent multimers of different FtsK domains (36). This protein was fused to His and FLAG tags at its N-terminal end for subsequent purification and western blot analysis (see ‘Materials and Methods’; Supplementary Figure S1).

We assayed the functionality of the 3γEc protein by testing the known activities of γEc. The induction of XerCD/dif recombination was tested using a Δ(lacI) E. coli strain carrying a dif-lacI-dif recombination cassette inserted in place of the dif site on the chromosome, which allows accurate measurement of recombination frequencies (29). This strain was rendered Δ(fisKγ), and the γEc or 3γEc protein were produced from a plasmid (see ‘Materials and Methods’). As previously reported, fisKγ deletion drastically reduced the recombination frequency compared to fisKwt and γEc production partially restored recombination (Figure 1C) (29). The 3γEc protein was readily produced in vivo at levels comparable to FtsKγEc alone in the same conditions (see ‘Materials and Methods’; Supplementary Figure S1) and had the same activity as γEc for the induction of XerCD/dif recombination (Figure 1C). These results are consistent with a recent report showing that covalent trimers of E. coli FtsKγ can induce XerCD/dif recombination in vitro and in vivo between plasmid-borne dif sites (37). We concluded that the 3γEc protein displays the same activity as FtsKγEc for the induction of XerCD/dif recombination.

The 3γEc protein was purified (see ‘Materials and Methods’; Supplementary Figure S1), and its capacity to bind KOPS-containing DNA was assayed in EMSA experiments. We used different DNA fragments containing either one KOPS or three non-overlapping KOPS 6 bp apart and assayed binding at two 3γEc concentrations. This was done in the presence or not of a large excess of competitor DNA devoid of KOPS motifs (polyIdC). The single KOPS-containing DNA was slightly shifted after incubation with 3γEc, the 3γEc-DNA complexes not migrating as a single shifted band but forming a smear immediately up to the free DNA (Figure 1D). This smear disappeared in the presence of competitor DNA. These results are reminiscent of the poor binding efficiency observed using a purified FtsKγ monomer and a DNA containing three overlapping KOPS (27). In contrast, binding of 3γEc to a DNA containing three non-overlapping KOPS was clearly detectable and formed a major complex in the absence of competitor DNA (Figure 1E). This complex appeared unstable during migration but formed efficiently even in the presence of competitor DNA (Figure 1E, right). These results combined with previously reported data show that the FtsKγ-KOPS interaction is poorly efficient and forms unstable complexes that are dissociated during migration in EMSA experiments and are displaced by an excess of non-specific DNA. FtsKγ-KOPS complexes are nevertheless readily detected in EMSA experiments using the 3γEc protein, which renders possible the characterization of KOPS from different species using this in vitro assay.

Lactococcus lactis FtsKγ does not recognize KOPS or AIMS motifs

We constructed a gene coding for a chimera protein, 3γLl, equivalent to 3γEc but containing three copies of the FtsKγLl subdomain (see ‘Materials and Methods’). The 3γLl protein was produced at quantities equivalent to 3γEc (Supplementary Figure S1). 3γLl was purified and we assayed binding to the DNA containing three E. coli KOPS (Figure 1F). No binding was detectable in conditions where the 3γEc-KOPS complexes are readily detected (compare with Figure 1E). This suggested that L. lactis FtsK does not recognize E. coli KOPS. This hypothesis was consistent with the distribution of E. coli KOPS on the L. lactis chromosome (Figure 2). Whereas KOPS are numerous and highly skewed on the E. coli chromosome, they are infrequent and poorly skewed on the L. lactis genome. From these criteria, the SRS motif appeared as even worse candidate than KOPS to fulfil the role of KOPS in L. lactis (Figure 2).

We next assayed the four AIMS motifs reported for L. lactis (6). We assayed binding of the 3γLl protein to four different DNA fragments, each containing three copies of a particular AIMS motif. Three of the four fragments yielded no detectable binding (Supplementary Figure S2). The fourth fragment, containing three consecutive 5′-AAGAAGAT-3′ motif, was reproducibly slightly shifted by 3γLl (Supplementary Figure S2). This
binding activity, however, appeared largely weaker than binding of the $3\gamma_{Ec}$ protein to a DNA containing three KOPS (compare Supplementary Figure S2 with Figure 1E). This weak binding may be due to a faint activity of the $3\gamma_{Ll}$ protein compared to its E. coli counterpart. Alternatively, the L. lactis KOPS motif may differ from both known KOPS and AIMS motifs. To differentiate between these two hypotheses, we attempted to improve KOPS prediction and find better candidate motifs in the L. lactis genome.

**Definition of prediction criteria for KOPS**

We reasoned that the common properties of KOPS and SRS motifs distribution in their respective genomes should allow us to establish prediction criteria for KOPS in...
L. lactis. Since both KOPS and SRS are octamers and the data available for motifs consensus show that the E. coli KOPS is degenerated at least at the fourth position, we analysed all families of octamers degenerated at one of any positions (see ‘Materials and Methods’; Table 1). As previously shown (2,8), KOPS and SRS motifs are significantly over-represented and skewed, with more than 75% present on the leading strand (Table 1). Indeed, a combination of the over-representation and skew scores identified the E. coli KOPS motif as one of the five most exceptional motifs [(2); Supplementary Figure S3A]. However, the same criteria did not discriminate clearly enough the KOPS motif in V. cholerae and the SRS motif in B. subtilis from all other octamers (Supplementary Figure S3B and S3C), suggesting that additional criteria are necessary to de novo predict KOPS motifs.

As E. coli FtsK acts mainly in a ~350-kb region around dif that represents ~7% of its genome (21), we speculated that KOPS distribution might be particularly important in this region. We looked for specific properties of KOPS/SRS in the equivalent region (here called the dif region) in V. cholerae and B. subtilis genomes. The skew of KOPS and SRS motifs, already high on the whole genome, was even higher in the dif region where ~90% of them were on the leading strand (Table 1). They also show an increased frequency (higher than 1/16 kb) and are significantly over-represented in this region (Table 1). This suggested that criteria for prediction of KOPS motifs should include a minimal leading strand skew in the dif region as well as a minimal frequency in this region. We chose as selective criterion a minimal skew of 90% in the dif region, because this is the most important property of KOPS and SRS motifs with respect to their activity. The minimal frequency was set conservatively at 1 motif every 40 kb because frequency is less critical to KOPS activity. Analyzing all octamers in E. coli, V. cholerae

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**Table 1. Properties of known KOPS motifs**

<table>
<thead>
<tr>
<th>Species (Motif)</th>
<th>Region analysed</th>
<th>Leading strand skew</th>
<th>Over-representation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Skew(^a)</td>
<td>P-value(^b)</td>
</tr>
<tr>
<td>E. coli GGGNAGGG</td>
<td>Complete genome</td>
<td>0.91</td>
<td>7.72 × 10(^{-24})</td>
</tr>
<tr>
<td></td>
<td>dif region</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>V. cholerae chr. 1 GGGNAGGG</td>
<td>Complete genome</td>
<td>0.80</td>
<td>3.85 × 10(^{-5})</td>
</tr>
<tr>
<td></td>
<td>dif region</td>
<td>0.92</td>
<td>0.054</td>
</tr>
<tr>
<td>B. subtilis GAGNAGGG</td>
<td>Complete genome</td>
<td>0.79</td>
<td>5.83 × 10(^{-4})</td>
</tr>
<tr>
<td></td>
<td>dif region</td>
<td>0.90</td>
<td>0.07</td>
</tr>
</tbody>
</table>

\(^a\)The skew is the ratio of number of motifs on the leading strand to total number of motifs.
\(^b\)The \(P\)-value evaluates the probability that the observed skew is explained by chance.
\(^c\)Skew rank: all motifs are ranked according to their skew significance: the lower the rank, the more significantly skewed the motif.
\(^d\)1/\(x\) kb correspond to the average frequency of motifs in the region of interest expressed as 1 motif per \(x\) kilo-base.
\(^e\)The frequency \(P\)-value evaluates the probability that the observed frequency is explained by chance.
\(^f\)Over-representation rank: all motifs are ranked according to their over-representation: the lower the rank, the more over-represented the motif.

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L. lactis. Since both KOPS and SRS are octamers and the data available for motifs consensus show that the E. coli KOPS is degenerated at least at the fourth position, we analysed all families of octamers degenerated at one of any positions (see ‘Materials and Methods’; Table 1). As previously shown (2,8), KOPS and SRS motifs are significantly over-represented and skewed, with more than 75% present on the leading strand (Table 1). Indeed, a combination of the over-representation and skew scores identified the E. coli KOPS motif as one of the five most exceptional motifs [(2); Supplementary Figure S3A]. However, the same criteria did not discriminate clearly enough the KOPS motif in V. cholerae and the SRS motif in B. subtilis from all other octamers (Supplementary Figure S3B and S3C), suggesting that additional criteria are necessary to de novo predict KOPS motifs.

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**Figure 2.** Escherichia coli KOPS and B. subtilis SRS motifs are bad candidate motifs for L. lactis KOPS motifs. The graphs show distribution of KOPS or SRS motifs in relevant bacterial genomes. Genomes and motifs are indicated. Coordinates are in bp. Grey arrowheads show the position of the chromosome dimer resolution site. The sequence is red on the top DNA strand; a +1 bar indicates a motif and a −1 bar its complementary sequence. Graphs were generated using an in-house version of the FindOligomers software (5).
and *B. subtilis* with these additional criteria allowed us to identify the KOPS and SRS motifs among the best candidates (Supplementary Figure S3D–S3F, respectively, where red dots respond to the *dif* region criteria). In addition, KOPS and SRS motifs were found among the most skewed motifs in the *dif* regions of the three genomes (reported as the intensity of red dots in Supplementary Figure S3D–S3F). We thus defined the following criteria for KOPS prediction: (i) a high skew and over-representation score on the whole genome, (ii) an occurrence of at least 1 every 40 kb in the *dif* region and (iii) a skew of at least 90% in the *dif* region with higher attention paid to the most skewed motifs.

**Prediction of KOPS candidates in *L. lactis***

We used the criteria defined above to predicted possible KOPS motifs in *L. lactis*. As KOPS and SRS motifs are octamers, we initially analysed the distribution of all octamers degenerated at one position on the leading strand of the *L. lactis* subsp. *lactis* IL1403 genome (see ‘Materials and Methods’, Figure 3A). The twenty best octamer candidates from our prediction criteria tended to be rich in purine bases (Supplementary Table 1). Interestingly, we noticed that 7 out of these 20 candidate motifs were composed of three very similar heptamer sub-motifs: 5′-GNAGAAG-3′, 5′-GANGAAG-3′ or 5′-GAAGNAG-3′ (Supplementary Table 1). This suggested that the *L. lactis* KOPS could be a heptamer. Indeed, when applying the same prediction criteria to all heptamers in this species, the motif 5′-GAAGAAG-3′ had a particularly striking distribution (Figure 3B): this motif is very frequent (1/2.1 kb), as it corresponds to the 10th most over-represented motif on the leading strand (*P*-value = 2.15 × 10⁻¹⁶⁰) and the fifth on the *dif* region. Its skew is also very high on the whole genome and >91% in the *dif* region.

To check if the properties of the 5′-GAAGAAG-3′ motif was conserved in bacteria related to *L. lactis*, we first analysed the genome of *L. lactis* subsp. cremoris MG1363, which displays an average of 15% DNA divergence with the *L. lactis* subsp. *lactis* genome (38), although its FtsKγ subdomain is strictly identical (Figure 1A). The 5′-GAAGAAG-3′ motif was the best KOPS candidate motif in *L. lactis* subsp. cremoris, strengthening our assumption that it might function as KOPS in *L. lactis*. We then analysed skewed heptamers in the genomes of other Streptococcaceae: *S. pneumoniae*, *S. agalactiae* and *S. pyogenes* (Supplementary Figure S5). These bacteria harbour similar FtsKγ subdomains that diverge from the *L. lactis* FtsKγ (Figure 1A). In these species, the 5′-GAAGAAG-3′ motif, although over-represented, was not skewed enough to fulfil our criteria. Furthermore, the sequence of the best candidate motifs varied between these three species: 5′-GCAGATG-3′ in *S. pneumoniae*, 5′-GAAGCAG-3′ in *S. agalactiae* and 5′-GTAGAAG-3′ in *S. pyogenes* (Supplementary Figure S5 and Supplementary Table S2). These motifs show a sequence related to but different from the 5′-GAAGAAG-3′ motif. This suggests that the KOPS
motif might be less conserved in *Streptococcaceae* compared to γ-proteobacteria.

*Lactococcus lactis* FtsKγ binds the 7 bp 5′-GAAGAAG-3′ motif

The above analysis showed that KOPS candidate motifs in *L. lactis* were 7 or 8 bp poly-purine tracks, a number of them containing the GAA trinucleotide. We therefore first assayed a DNA containing six consecutive GAA for 3γLl binding in EMSA experiments. This DNA was shifted efficiently by 3γLl even in the presence of competitor DNA (Figure 4A). In contrast, 3γEc barely bound the DNA containing consecutive GAA and only in the absence of competitor DNA. Thus, a DNA containing consecutive GAA trinucleotides is efficiently and specifically bound by 3γLl. We then directly assayed the 5′-GAAGAAG-3′ heptamer, which is the best candidate found in our predictive approach (Figure 3). 3γLl bound a DNA fragment containing a single 5′-GAAGAAG-3′ motif poorly in a similar manner that 3γEc bound a fragment containing a single KOPS (compare Figure 4B with 1D). We thus constructed a DNA containing three non-overlapping 5′-GAAGAAG-3′ motifs separated by 6 bp. 3γLl formed specific complexes with this DNA both in the absence and presence of competitor DNA (Figure 4C). We concluded that the 5′-GAAGAAG-3′ heptamer is sufficient for specific binding by the 3γLl protein. However, since KOPS and SRS motifs are 8 bp long, we considered the possibility that octamers may be better substrates than the 5′-GAAGAAG-3′ heptamer. We thus assayed

Figure 4. The 3γLl protein recognizes the 5′-GAAGAAG-3′ heptamer. (A–C) Same EMSA experiment as in Figure 1D–F, with DNA substrates containing a repetition of 5′-GAA-3′ motifs (A), a single 5′-GAAGAAG-3′ motif (B), or three non-overlapping 5′-GAAGAAG-3′ motifs (C) and the indicated proteins. The relevant DNA sequences are shown below the gels. (D–F) ITC experiments performed by titrating the 3γLl protein with the indicated DNA substrates: (D) containing a single 5′-GAAGAAG-3′ motif, (E) three non-overlapping 5′-GAAGAAG-3′ motifs and (F) a single *E. coli* KOPS (5′-GGGCAGGG-3′). The best-fitted model curves using a ‘one set of sites’ model are shown (continuous line) with the corresponding stoichiometry values indicated (N).
of L. lactis together, these data strongly suggest that the experiments, we measured the 3G-3 motif that is not recognized by 3γLl; (ii) the 3γLl binding sequence is the 5'-GAAGAAG-3' heptamer since the three possible octamers containing this motif and contained into the 5'-GAA-3' concatamer shown in Figure 4 are not better binding sites. Both the sequence and the shorter length of the 3γLl binding motif compared to P. aeruginosas and E. coli KOPS suggest a different mode of binding. Indeed, in the P. aeruginosas FtsKγ/KOPS complex, the three FtsKγ monomers are located head to tail. Two monomers recognize the two GGG repeats of the KOPS motif, while the third appears to stabilize the complexes by protein–protein interaction and may recognize the central NA (26). The shorter length of the L. lactis motif would imply a different geometry for FtsKγ monomers arrangement in the complex. In addition, the absence of direct repetition of base triple at the edges of the 5'-GAAGAAG-3' motif appears inconsistent with the mode of binding described for P. aeruginosas.

Since the interaction of 3γLl with a single 5'-GAAGAA G-3' motif appears unstable and poorly specific in EMSA experiments, we measured the 3γLl/5'-GAAGAAG-3' interaction using isothermal titration calorimetry (ITC; see ‘Materials and Methods’). Results showed that 3γLl formed stable complexes with DNA containing either one or three 5'-GAAGAAG-3' motifs (Figure 4D and E). The patterns obtained fitted well with a DNA/protein stoichiometry 1:1 and 3:1 for DNA containing one and three 5'-GAAGAAG-3' motifs, respectively. This indicates that one molecule of 3γLl binds to one 5'-GAAGAAG-3' motif (therefore, three molecules of 3γLl associate with one DNA molecule containing three motifs). In contrast, the ITC data resulting from the titration of 3γLl by a DNA containing one E. coli KOPS motif failed to indicate any binding (Figure 4). Taken together, these data strongly suggest that the γ subdomain of L. lactis FtsK specifically recognizes the 5'-GAAGAA G-3' motif.

The 5'-GAAGAAG-3' motif controls FtsK translocation in vivo
To assay the activity of the 5'-GAAGAAG-3' motif in vivo, we took advantage of the role of FtsK in the induction of XerCD/dif recombination. We have previously reported that inserting three consecutive KOPS in non-permissive orientation next to a dif site lowers its capacity to recombine in E. coli (29). Non-permissive KOPS are thought to promote FtsK loading and subsequent translocation away from dif thereby lowering its capacity to reach the XerCD/dif complex (26). We also previously constructed an E. coli strain carrying the C-terminal part of L. lactis ftsK in place of its E. coli counterpart (Figure 5A). The resulting strain fully supported resolution of chromosome dimers, making this strain a useful tool to study FtsKCLl activities in a cellular context (29). To assay the effect of the 5'-GAAGAAG-3' motif on FtsK activity, we constructed a set of strains carrying either the E. coli ftsK or ftsKCLl gene and non-permissive E. coli KOPS (5'-GGGCAAGG-3') or 5'-GAAGAAG-3' motifs next to a dif site of a dif-lacI-dif construct inserted in place of the dif site (Figure 5B). Recombination was scored as the appearance of dark blue colonies on indicator medium-containing plates [see ‘Materials and Methods’; (29)].
shown, insertion of three non-permissive KOPS lowered E. coli FtsK-driven recombination about 2-fold (Figure 5B). A single non-permissive Escherichia coli KOPS yielded no significant effect in this assay. No significant effect of non-permissive 5'-GAAGAAG-3’ motif was detected on E. coli FtsK-driven recombination, showing that this motif has no KOPS activity on E. coli FtsK. On the other hand, E. coli KOPS had no effect on FtsKCLl-driven recombination, showing that E. coli KOPS do not control L. lactis FtsK translocation (Figure 5). As in the E. coli FtsK/KOPS system, a single non-permissive 5'-GAAGAAG-3’ motif had no significant effect on FtsKCLl-driven recombination frequencies. However, three consecutive 5'-GAAGAAG-3’ motifs lowered recombination frequencies almost 100 times (Figure 5, last line, compare with the two time effect yielded by E. coli KOPS). This high level of inhibition might reflect a higher efficiency of L. lactis KOPS compared to E. coli KOPS and/or a lower activity of the FtsKCLl protein compared to E. coli FtsK. These results show that the 5’-GAAGAAG G-3’ motif controls L. lactis FtsK translocation in a cellular context.

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